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Promoter usage and alternative splicing

Alberto R Kornblihtt

Recent findings justify a renewed interest in alternative splicing (AS): the process is more a rule than an exception as it affects the expression of 60% of human genes; it explains how a vast mammalian proteomic complexity is achieved with a limited number of genes; and mutations in AS regulatory sequences are a widespread source of human disease. AS regulation not only depends on the interaction of splicing factors with their target sequences in the pre-mRNA but is coupled to transcription. A clearer picture is emerging of the mechanisms by which transcription affects AS through promoter identity and occupation. These mechanisms involve the recruitment of factors with dual functions in transcription and splicing (i.e. that contain both functional domains and hence link the two processes) and the control of RNA polymerase II elongation.

Addresses
Laboratorio de Fisiologia y Biologia Molecular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria — Pabellon 2 — 2° piso, (C1428EHA) Buenos Aires, Argentina

Corresponding author: Kornblihtt, Alberto R (ark@fbmc.fcen.uba.ar)

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Introduction

Promoters and enhancers are *cis*-acting elements that control gene transcription via complex networks of protein–DNA and protein–protein interactions. Although only promoters can actually recruit RNA polymerases to genes, both enhancers and promoters can control transcriptional initiation and elongation. The idea that the regulatory mechanisms affecting these gene elements only controlled the quantity and not the identity of their corresponding transcripts dominated our conception of gene expression for decades. Indeed, transcription and pre-mRNA processing were thought to be independent events until a series of biochemical, cytological and functional experiments demonstrated that all three processing reactions (capping, splicing and cleavage/polyadenylation) can be tightly coupled to RNA polymerase II (pol II) transcription [1–5]. This review focuses on recent evidence revealing how pol II promoter structure and occupation by transcription factors modulates alternative splicing (AS), strengthening the concept of a physical and functional coupling between transcription and splicing.

The importance of being a pol II promoter

The facts that only protein-encoding genes encode pre-mRNAs subjected to the above-mentioned processing reactions and that these genes are exclusively transcribed by RNA polymerase II are *per se* suggestive of a linking mechanism. When a protein-encoding gene is put under the control of a RNA polymerase I promoter, transcription is efficiently performed by pol I, but the resulting transcripts are not polyadenylated and become extremely unstable *in vivo* [6]. Similarly, transcripts produced *in vivo* by hybrid genes containing RNA polymerase III promoters fused to sequences that are normally transcribed by pol II are poorly spliced and polyadenylated [7]. Moreover, when pol II genes are put under the control of the T7 polymerase promoter and transcribed *in vivo* by the prokaryotic polymerase ectopically expressed in eukaryotic cells, pre-mRNA processing is also affected [8,9]. These findings appear rather difficult to reconcile with the fact that splicing and cleavage/polyadenylation can be duplicated in cell-free extracts when an *in vitro*-synthesized, pure and full length pre-mRNA is provided as substrate. This post-transcriptional, *in vitro* processing strategy has been crucial to our efforts to decipher the chemistry and molecular participants of the processing reactions. However, its extensive and successful use has diverted our attention from the *in vivo* situation, where processing is linked to transcription.

What makes pol II promoters permissive for correct pre-mRNA processing? The answer is that the large subunit of this enzyme possesses a distinctive C-terminal domain (CTD) composed of 52 tandem repeats in mammals (26 in yeast) of the consensus heptad YSPTSPS. The CTD serines at positions 2 and 5 are subjected to regulatory phosphorylations. Phosphorylation of Ser5 by TFIIH is linked to transcriptional initiation, whereas phosphorylation of Ser2 by P-TEFb is associated with transcriptional elongation. Truncation of the CTD causes defects in capping, cleavage/polyadenylation and splicing [10] whereas isolated CTD fragments are able to activate splicing *in vitro* [11]. Not only does the CTD act as a landing pad for processing factors [1], but dynamic changes in CTD structure and phosphorylation play significant roles in RNA processing. For instance, the peptidyl-prolyl isomerase Pin 1, which stimulates CTD phosphorylation by cdc2/cyclin B and hence affects CTD structure, inhibits pol II-dependent splicing *in vitro*

[12]. Inhibition of P-TEFb-mediated CTD phosphorylation inhibits co-transcriptional spicing and 3′-end formation *in vivo* in *Xenopus* oocytes. By contrast, processing of injected pre-mRNA is unaffected by P-TEFb kinase inhibition, which strongly indicates that pol II does not participate directly in post-transcriptional processing, but that phosphorylation of its CTD is required for efficient co-transcriptional processing [13•]. New insights into the mechanism by which the CTD functions in splicing come from recent *in vitro* experiments with a protein in which the pol II CTD was fused at the C terminus of the splicing factor SF2/ASF (ASF–CTD). Compared to SF2/ASF alone, ASF–CTD increased the reaction rate during the early stages of splicing. Both the RNA-targeting domain of SF2/ASF and phosphorylation of the CTD moiety were necessary for the stimulation of splicing by the chimeric protein [14•].

The importance of being a particular pol II promoter

A finding that strengthened the concept of coupling between the transcription and splicing machineries was that differences in pol II promoter structure lead to differences in AS of the transcript [15,16]. The system that was analyzed involved transient transfection of mammalian cells with AS reporter minigenes carrying the extra domain I (EDI) exon, which encodes a facultative repeat of fibronectin (FN) under the control of different pol II promoters. EDI contains an exonic splicing enhancer (ESE), which is targeted by the splicing factors SF2/ASF and 9G8. When transcription is driven by the α-globin promoter, for example, EDI inclusion levels in the mature mRNA are ~10 times lower than when transcription is driven by the FN or cytomegalovirus (CMV) promoters.

These effects are not the trivial consequence of different mRNA levels produced by each promoter (i.e. promoter strength), but depend on some qualitative properties conferred by promoters to the transcription/RNA-processing machinery. This is consistent with recent microarray studies indicating that although, like global transcription profiles, global AS profiles reflect tissue identity, transcription (evaluated as promoter usage and strength) and AS act independently on different sets of genes to define tissue-specific expression profiles [17•].

Similar effects of promoters on AS have been found independently in other genes. Reporter minigenes whose products are subject to AS decisions in the CD44 and the calcitonin-gene-related product (CGRP) genes were put under the control of either steroid-sensitive or steroid-insensitive promoters. Steroid hormones affected splice site selection only of pre-mRNAs produced by the first type of promoters. As in the case of FN EDI, promoter-dependent hormonal effects on splicing were not a consequence of an increase in transcription rate or saturation of the splicing machinery [18]. Promoter-dependent AS patterns have been also found in the cystic fibrosis transmembrane regulator [19] and in the fibroblast growth factor receptor *2* genes [20].

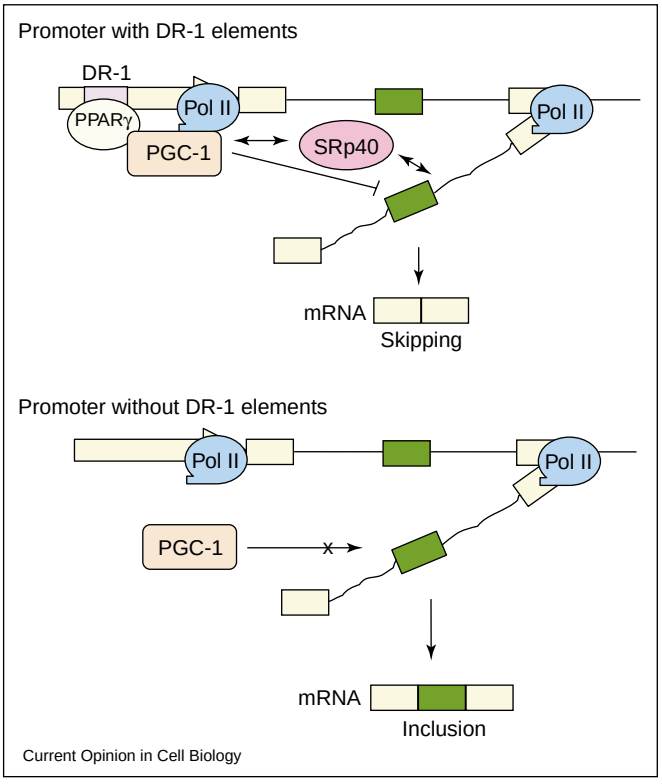
The finding that promoter structure is important for AS suggests that factors that regulate AS could be acting through promoters and that cell-specific AS may not simply result from the differential abundance of ubiquitous SR proteins, but from a more complex process involving cell-specific promoter occupation. However, promoters are not swapped in nature and, as most genes have a single promoter, the only conceivable way by which promoter architecture could control AS *in vivo* would be the differential occupation of promoters by transcription factors with different types of activation domains and/or mechanistic properties. Accordingly, it has been found that transcriptional activators with different actions on pol II initiation and elongation affect AS differentially [21] (see below).

Transcriptional co-regulators have been also implicated in the control of AS. Several co-regulators of steroid hormone nuclear receptors showed differential effects on AS in a promoter-dependent manner [22]. Some co-regulators act by recruiting coactivators, such as the coactivator CoAA (coactivator activator), a protein that interacts with the transcriptional coregulator TRBP, which is in turn recruited to promoters through interactions with activated nuclear receptors. CoAA regulates AS in a promoter-dependent manner. It similarly enhances transcriptional activities fired by the steroid-sensitive or -insensitive promoters, but only affects AS of transcripts synthesized from the progesterone-activated MMTV promoter [23]. In addition, transcriptional activators seem to modulate not only AS but also constitutive splicing in a pol II-CTD-dependent manner [24].

Factor recruitment

A possible mechanism that would explain the promoter effect is that the promoter itself is responsible for recruiting splicing factors, such as SR proteins, to the site of transcription, possibly through transcription factors that bind the promoter or the transcriptional enhancers. Some proteins, for example the above-mentioned synthetic chimera ASF–CTD [14•], naturally display dual functions, acting in both transcription and splicing. Good examples are the transcriptional activator of the human papilloma virus [25] and the thermogenic coactivator PGC-1. Interestingly, PGC-1 affects AS, but only when it is recruited to complexes that interact with gene promoters [26] (Figure 1). Other mammalian cell candidates include the product of the WT-1 gene, which is essential for normal kidney development [27]; SAF-B, which mediates chromatin attachment to the nuclear matrix [28]; CA150, a human nuclear factor with characteristic WW and FF domains implicated in transcrip-

Figure 1



Example of how promoters may affect alternative splicing (AS) through recruitment of factors with dual functions in transcription and splicing (i.e. that contain both functional domains and hence link the two processes); based on [26]. A promoter with a DR-1 element binds the transcription factor PPARγ, which in turn recruits the transcriptional coactivator PGC-1. PGC-1 interacts with pol II and other proteins of the pre-initiation complex as well as with the splicing factor SRp40, which controls inclusion of the fibronectin EDII (also known as EDB or EIIIB) alternative exon (dark green). PGC-1 inhibits inclusion of EDII into the mature mRNA, only when targeted to a promoter.

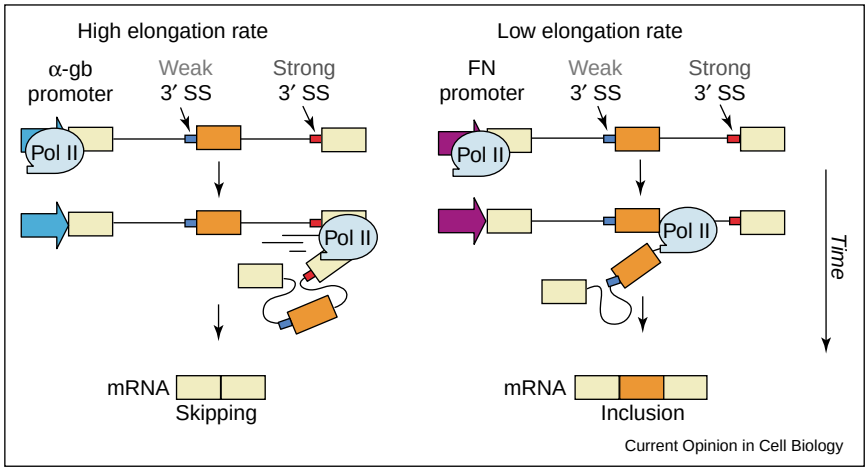
tional elongation [29,30]; and a group of proteins known as SCAFs (SR-like CTD associated factors), which interact with the CTD and, similarly to SR proteins, contain an RS domain and an RNA-binding domain [31]. Two budding-yeast CTD-interacting proteins could be added to the list: the splicing factor Prp40 [32] and Ess1, a peptidyl prolyl isomerase, proposed to act in *cis*/*trans* protein isomerizations that could play a crucial role in the recognition of CTD by other proteins [33].

Pol II elongation

Promoters can also control AS via the regulation of pol II elongation rates or processivity. Low pol II elongation rates or internal pauses for elongation would favor the inclusion of alternative exons governed by an exon skipping mechanism, whereas a highly elongating pol II, or the absence of internal pauses, would favor exclusion of these kinds of exons. The elongation rate affects EDI splicing as a consequence of EDI pre-mRNA sequence. EDI exon skipping occurs because the 3′ splice site of the upstream intron is suboptimal compared to the 3′ splice site of the downstream intron. If the polymerase pauses anywhere between these two sites, only elimination of the upstream intron can take place. Once the pause is passed or the polymerase proceeds, there is no option for the splicing machinery but to eliminate the downstream intron, which leads to exon inclusion. A highly processive elongating pol II, or the absence of internal pauses, would favor the simultaneous presentation of both introns to the splicing machinery, a situation in which the stronger 3′ splice site of the downstream intron out-competes the weaker 3′ splice site of the upstream intron, resulting in exon skipping. When a weak 3′ splice site is followed by a strong one, as seen in many examples of AS, pol II elongation rates affect the relative amounts of splicing isoforms (Figure 2). In contrast, when two consecutive strong 3′ splice sites occur, as in constitutive splicing, pol II elongation rates are irrelevant.

A kinetic aspect to the effect of transcription on splicing was originally suggested by Eperon *et al.* [34], who found that the rate of RNA synthesis affects its secondary structure, which in turn affects splicing. A similar mechanism involving a kinetic link between transcription and splicing was suggested from experiments in which pol II pause sites affect AS by delaying the transcription of an

Figure 2



Example of how promoters may affect alternative splicing (AS) through the control of pol II elongation rates (based on [21,36,39,43••,44]). The 3′ splice site (SS) by the alternative fibronectin (FN) EDI exon (orange) is weaker than the 3′ SS of the downstream intron. Low transcriptional elongation rates such as those elicited by the FN promoter (right) favor exon inclusion, whereas high elongation rates such as those elicited by the α-globin promoter (left) favor skipping.

essential splicing inhibitory element (DRE) required for regulation of tropomyosin exon 3 [35].

Several experiments, of different nature, support indirectly a role for pol II elongation in alternative splicing

Transcription factors that stimulate mostly transcriptional initiation, such as Sp1 and CTF/NF1, have little effect on AS, whereas factors that stimulate elongation, such as VP16, provoke skipping of the EDI exon [21,36].

Phosphorylation of pol II CTD at serine-2 by the elongation factor P-TEFb converts the polymerase from a non-processive to a processive form. Inhibitors of this kinase such as DRB (dichlororibofuranosylbenzimidazole) inhibit pol II elongation. Cells transfected with EDI splicing reporters and treated with DRB displayed a threefold increase in EDI inclusion into mature mRNA compared to untreated cells [21].

Changes in chromatin structure also affect splicing. Trichostatin A, a potent inhibitor of histone deacetylation, favors EDI skipping [21]. This supports the hypothesis that acetylation of the core histones would facilitate the passage of the transcribing polymerase, which is in turn consistent with the proposal that chromatin opening is mediated by DNA tracking by a transcribing pol II complex piggybacking a histone acetyltransferase activity [37]. Consistently, replication of the transfected minigene reporters, after which these template plasmids adopt a more compact chromatin structure, causes a 10–30-fold increase in EDI exon inclusion levels in the transcript, independently of the promoter used [36]. Interestingly, it was recently found that intragenic DNA methylation provokes a close chromatin structure and subsequently reduces the efficiency of pol II elongation [38••]. This suggests that CpG islands might be involved not only in transcriptional silencing when located at promoter regions but also in the regulation of splicing via elongation when located at regions downstream of the promoter.

Transcriptional regulatory elements, such as the SV40 enhancer, that activate pol II elongation provoke skipping of the EDI exon, also independently of the promoter used [39].

Chromatin immunoprecipitation experiments reveal stalling of pol II molecules upstream of the alternative EDI on minigenes with promoters that favor EDI inclusion (i.e. the FN promoter) compared to minigenes with promoters that favor EDI skipping (i.e. the α-globin promoter [39].

Mutation analysis shows that the better an alternative exon is recognized by the splicing machinery, the less its degree of inclusion is affected by factors that modulate transcriptional elongation [40]. This indicates that the promoter control is not obligatory and must coexist with other important AS regulatory mechanisms [41,42].

Slow polymerases and alternative splicing

A more direct proof for the elongation mechanism in the transcriptional control of AS in human cells was provided by the use of a mutant form of pol II (called C4) with a lower elongation rate [43••]. The slow polymerase increases the inclusion of the fibronectin EDI exon threefold, confirming the hypothesis that there is an inverse correlation between elongation rate and inclusion

of this alternative exon. The C4 mutation also affected the splicing of Adenovirus E1a by favoring the use of the most upstream of the three alternative 5′ splice sites that compete for a common 3′ splice site. Most importantly and of physiological relevance, *Drosophila* flies carrying the C4 mutation show changes in the AS profile of the large ultrabithorax (*Ubx*) endogenous gene. The observed changes are consistent with a kinetic mechanism that allows more time for early splicing events. Most interestingly, *Drosophila* that are heterozygous for the C4 allele but have two wild-type *Ubx* alleles show a mutant phenotype called '*Ubx* effect' that resembles the phenoptype of flies haploinsufficient for the Ubx protein.

Similar effects of pol II elongation rates on splicing were found in yeast. Alternative splicing is a very rare event in yeast. By mutating the branchpoint upstream of the constitutive internal exon of the *DYN2* gene, an artificial cassette exon that becomes alternatively spliced was created. Skipping of this exon is prevented when expressed in a yeast mutant carrying a slow pol II or in the presence of elongation inhibitors [44•]. This supports the hypothesis that the most important factor affecting the balance between exon skipping and exon inclusion is the relative rates of spliceosome formation and pol II processivity.

Alternative promoter usage and alternative splicing

Many eukaryotic genes contain multiple promoters, each subjected to different regulatory factors. By definition, each promoter determines a different start site and first exon and, in consequence, a different transcript. Frequently, transcripts arising from genes with multiple promoters only differ in their 5′ non-coding regions, sharing the same open reading frames. Alternative promoter usage is strongly linked to AS of internal exons and often has physiological implications, as is the case for the acetylcholinesterase [45] and Bcl-x [46] genes. It would be tempting to interpret the link between alternative promoter usage and splice site selection as a paradigmatic example of transcription/splicing coupling through either factor recruitment or pol II elongation. However, such a coupling mechanism remains to be proved, and any supporting evidence should first rule out the possibility that internal AS variants are the consequence of important changes in pre-mRNA secondary structure resulting from different first exon sequences.

Promoters at the end

A new dimension in gene regulation that might be related to the role of promoters in pre-mRNA processing emerges from findings that two particularly long genes of *Saccharomyces cerevisiae* exist in a looped conformation [47••]. The 'glue' that seems to keep their promoter and terminator regions in a close spatial proximity contains the pol II molecule itself, phosphorylated at its Ser5. Loss of Ser5 phosphorylation leads to the loss of the loop conformation. It will be extremely interesting to know whether gene loops exist in mammalian genes and, if so, whether they are related to the promotion of efficient transcriptional elongation as suggested for yeast.

Conclusions

The realization that capping, splicing, 3′ end formation, termination and mRNA export are coupled to transcription has given us a more dynamic and integrated view of the basics of eukaryotic gene expression. Because each process is extremely complex in itself, one should be cautious when attempting to simplify or generalize, bearing in mind that certain mechanisms might apply to a particular gene or set of genes but not to others.

In the case of AS, a complex panorama emerges when trying to summarize the factors involved in its regulation. On the *cis* side, we should not only take into account the specific sequences acting at the RNA level (splice sites, splicing enhancers and silencers, and determinants of pre-mRNA secondary structures) but also those acting at the DNA level such as promoters, transcriptional enhancers and the pol II pausing architecture [38••] of a gene. On the *trans* side, the abundance, cell localization and phosphorylation state of SR and hnRNP proteins should be complemented with those of transcription factors, co-activators, chromatin factors, CTD kinases, transcriptional elongation factors and factors with dual activities in both transcription and splicing.

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